University of Nevada, Reno

Inducible Cre-mediated excision yields incomplete *Ano1* knockdown in the murine gastrointestinal tract

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by

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BACHELOR OF SCIENCE

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Abstract

Anoctamin-1 (ANO1) is a calcium-activated chloride channel that is highly expressed in the muscle layer of the murine gastrointestinal (GI) tract, particularly interstitial cells of Cajal (ICC) responsible for regulation of GI contraction. The particular function of ANO1 in the GI tract or in ICC, however, has been characterized to a limited extent. Knockouts of *Ano1* – the gene precursor for ANO1 – in mouse models by classic breeding schemes are typically fatal by Day 8, so a novel method of *Ano1* elimination in the GI tracts of fully developed mice would facilitate further characterization of its function. In this study, inducible Cre-mediated excision resulted in significant *Ano1* reduction in several GI tissues of adult mice, although significant knockdown was not observed in the GI vasculature.

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Introduction

Interstitial cells of Cajal (ICC) form a network in the smooth muscle layer of the gastrointestinal tract, where they have been shown to be responsible for pacemaking activities (phasic, slow wave contractions) (Sanders, et al. 2006). In addition to this function, ICC play a major role in several functional gastrointestinal disorders related to problems in motility (Mostafa, et al. 2010). Anoctamin-1 (ANO1) – the product of the gene *Ano1* in mice, also known as transmembrane protein 16A (TMEM16A) – has been shown to act as a calcium-activated chloride channel in several tissues, and its role is crucial in normal murine development (Caputo, et al. 2008). In particular, *Ano1* is highly expressed in ICC within the gastrointestinal muscle layer, and is necessary for proper slow wave activity, although its particular function in ICC has yet to be fully characterized (Mazzone, et al. 2012; Hwang, et al. 2009). For instance, substantial *Ano1* gene expression in ICC that do not conduct slow waves suggests that ANO1 may have additional roles, namely in cell proliferation and tumorigenesis (Stanich, et al. 2011). A more complete and thorough classification of ANO1 function has potential implications in the characterization and treatment of gastrointestinal diseases, especially those related to gastrointestinal motility and tumorigenesis.

The function of ANO1 remains uncertain to date, largely because complete murine *Ano1* knockout by conventional breeding schemes is invariably fatal, leading to enhanced tumorigenesis and improper development of physical structures, with death occurring typically by Day 8 (Duvvuri, et al. 2012, Rock, et al. 2008). Although molecular characteristics of the *Ano1* gene and its alternative splicing transcripts have been described (O'Driscoll, et al. 2011), classic knockout techniques may only be used to

study ANO1 function in developing mice and in a limited set of tissues. Conventional knockouts have thus far not been particularly useful in determining ANO1 function in the gastrointestinal ICC network. ANO1 knockdown in fully developed wildtype mice would possibly bypass this limitation.

Cre/loxP recombination is a system that was identified in Bacteriophage P1 – an *E. coli* infecting phage – as a normal part of its lifestyle, and its use for genome manipulation techniques in mammalian cell lines and in live mice has been documented in the past (Sauer and Henderson 1998, Lallemand, et al. 2012). Essentially, this system of site-specific DNA recombination allows for the manipulation of a gene of interest – which has been "floxed," or transgenically flanked by *loxP* sites; these 34-bp sequences, containing an 8-bp "core" sequence, are targeted for excision by a Cre recombinase, which can be transgenically incorporated into host genomes (Sauer and Henderson 1998, Gu, et al. 1993). The result of the Cre/loxP recombination system depends on the directionality of the flanking *loxP* sites, with deletion occurring when the *loxP* sites are in the same directionality (Sauer, 2002). In the past, Cre/loxP recombination has been used as a means to induce knockout of genes, especially those for which manipulations are typically deleterious (Ruzankina, et al. 2007).

In order to control the timing and location of this genome manipulation system, distinct Cre strains may be used in which the Cre gene is linked to a tissue-specific promoter whose activation is only achieved by pharmaceutical administration (Sauer, 1998). Unfortunately, although some ICC-specific Cre strains exist, strains that are specific for the gastrointestinal muscle layer have yet to be created, preventing thorough *Ano1* excision in the gastrointestinal tract via tissue-specific Cre-mediated excision. To

bypass this, the Cre strain used in this study is subject to control by an ubiquitin-C promoter, ideally resulting in its strong expression in all tissues. Additionally, the Cre gene has been linked to ERT2, the gene for human estrogen receptor 2, which has been modified to only bind analogs of human estrogen (Ruzankina, et al. 2007). In principle, injection of Cre-ERT2^{+} mice with a human estrogen analog such as tamoxifen induces Cre recombinase activity, by allowing the Cre-ERT2 fusion protein to enter the nucleus and catalyze deletion of the "floxed" gene of interest, in this case Exon 12 of *Ano1* (selected because its deletion eliminates ANO1 activity). In fact, this particular system has been used to conditionally knockout *Ano1* in the neural and cardiac tissues of adult mice, suggesting it could be adapted to other tissues (Rock 2008). Hence, Cre/LoxP recombination remains an alluring prospect for the conditional and inducible knockout of *Ano1* in the gastrointestinal ICC of adult mice.

In this experimental approach, mice were bred that contained an *Ano1* gene, "floxed" at Exon 12 for both alleles, as well as a Cre-ERT2 transgene controlled by an ubiquitin C-promoter. Injection with tamoxifen resulted in *Ano1* deletion in several tissues, although incomplete excision was often observed. To produce a more desirable result, alternate breeding schemes were designed. All breeding schemes analyzed exhibited incomplete excision in the gastrointestinal tract, although excision appeared to be more thorough with certain methods.

Materials and Methods

Breeding *Ano1*$^{lox/lox}$cre-ERT2^{+} mice

A breeding scheme was designed to produce *Ano1*$^{lox/lox}$cre-ERT2^{+} mice. These individuals contained a functional Cre gene and two "floxed" *Ano1* alleles (*Ano1*$^{lox/lox}$) (Figure 1). To achieve this genotype, hemizygote mice for the Cre transgene of B6.Cg-Tg(UBC-cre/ESR1)1Ejb/J (Cre-ERT2, Jackson Laboratories) were bred with homozygous *Ano1*$^{lox/lox}$ mice. Heterozygous *Ano1*$^{lox/+}$cre-ERT2^{+} offspring were then bred together to produce homozygous *Ano1*$^{lox/lox}$cre-ERT2^{+} mice. In order to determine individual genotypes at each generation, DNA was extracted from ear tissue via the HotSHOT genomic DNA isolation technique (BioTechniques 2000). The Cre-ERT2 locus was then analyzed according to the strain-specific protocol, published by Jackson Laboratories, while the *Ano1*$^{lox/lox}$ locus was analyzed by polymerase chain reaction (PCR) and gel electrophoresis using the following conditions (Figure 2):

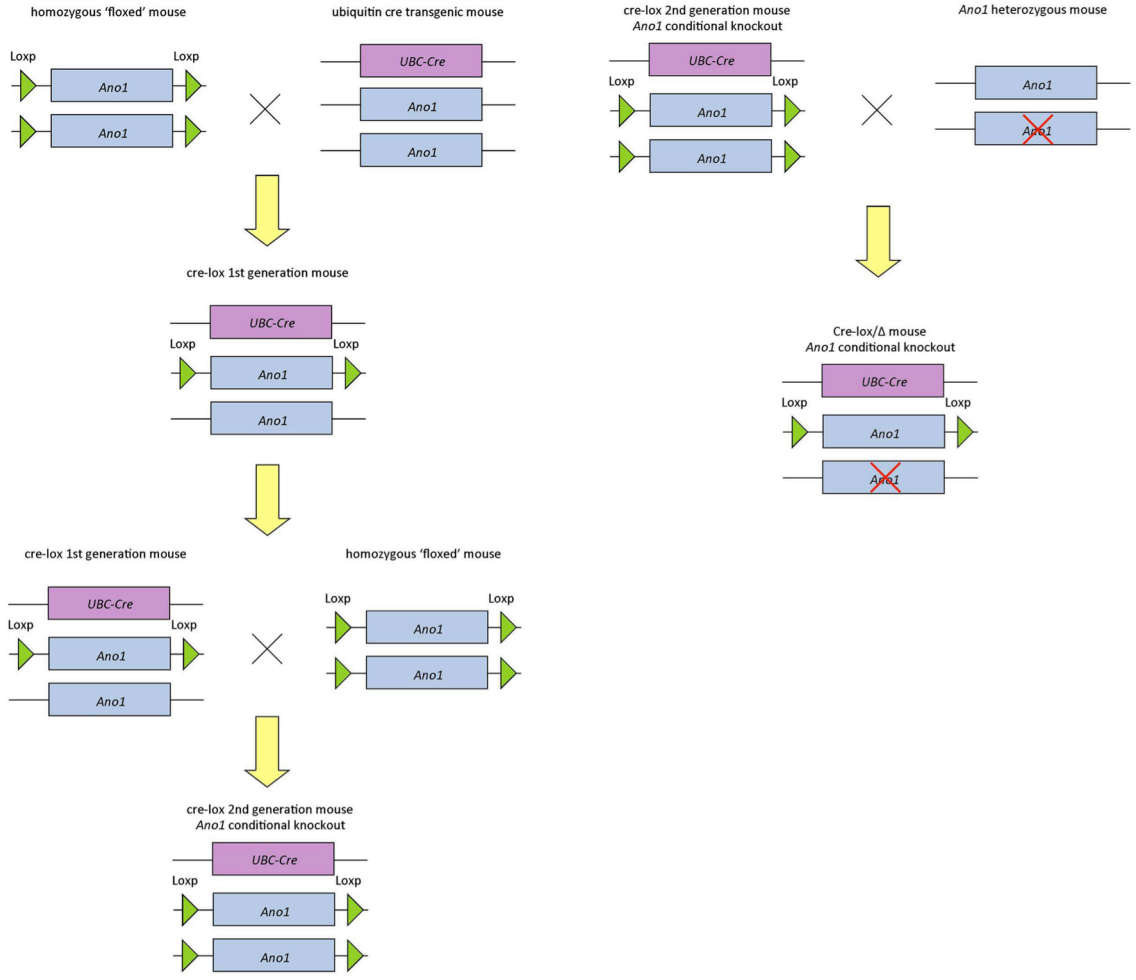

Figure 1. Different breeding schemes are implemented to produce an inducible *Ano1* knockout. Two different genotypes were produced by distinct breeding schemes. The first genotype – *Ano1*$^{lox/lox}$Cre-ERT2^{+} – requires excision of both *Ano1* alleles to produce a knockout. The second genotype – *Ano1*$^{lox/\Delta}$Cre-ERT2^{+} – only requires excision of a single *Ano1* allele, as the second allele has already been knocked out by conventional means.

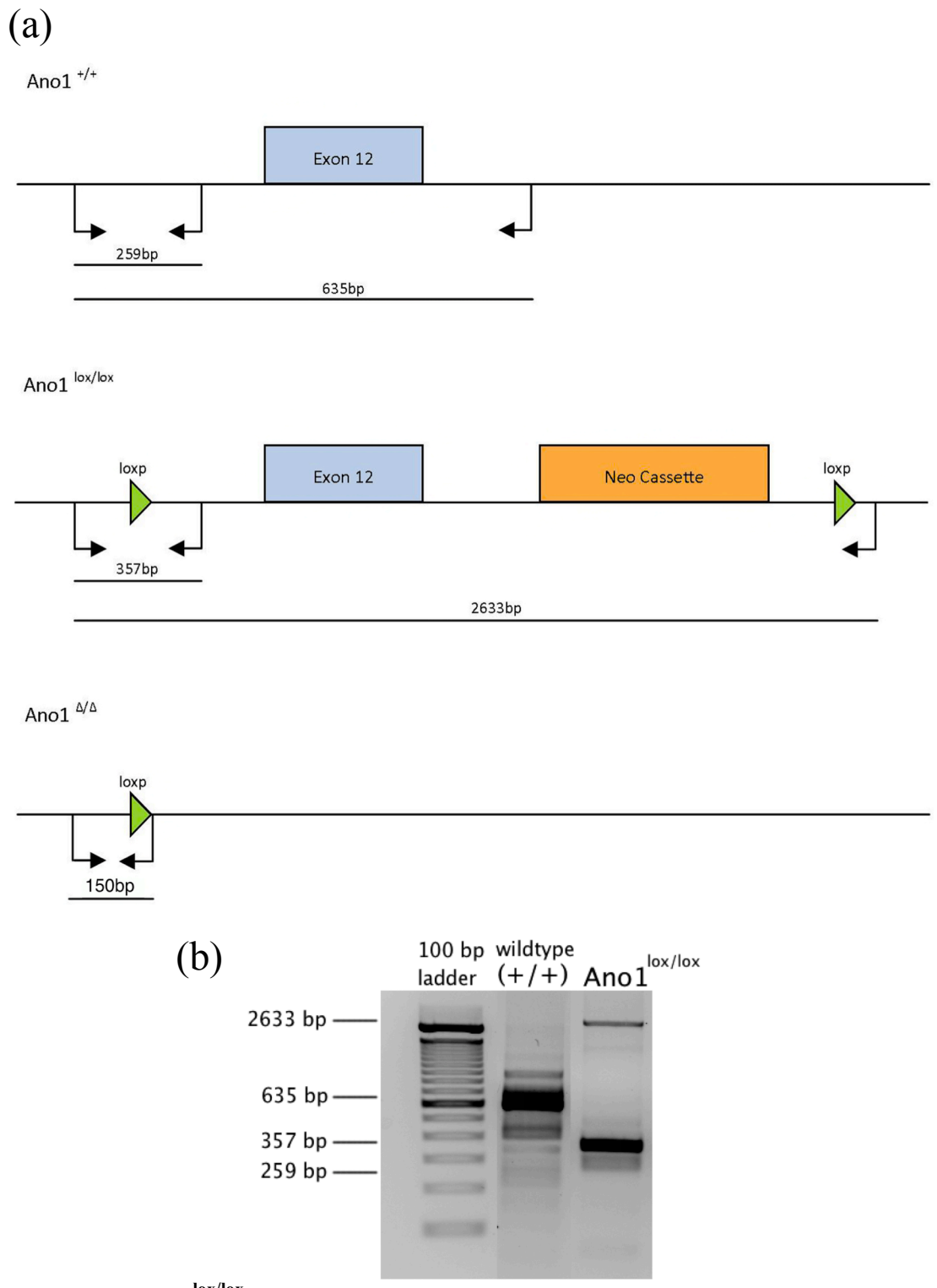

Figure 2. ***Ano1*$^{lox/lox}$ construct allows for selective determination of distinct genotypes.** **(a)** The *Ano1*$^{lox/lox}$ construct produces fragments varying in size between wildtype, lox/lox, and null alleles. **(b)** Genotypes prior to Cre-induction may be differentiated by PCR and gel electrophoresis, even with the presence of nonspecific fragments.

Forward Primer: ggctctatcaatgttctgttc

Reverse Primer R1: ctcaagtcctcaagtcccagtc

Reverse Primer R2: gagcggggttagaagatccttg

(Primers obtained from Eurofins MWG Operon.)

95°C for 2 min., 40 cycles (95°C for 15 s, 57°C for 2 min., 72°C for 30 s), 72°C for 4 min.

Induction of Cre activity

Cre activity was induced in adult mice to facilitate excision of Exon 12 of *Ano1* (Figure 3). Day 30 mice were injected intraperitoneally with a 20 mg/mL solution of tamoxifen

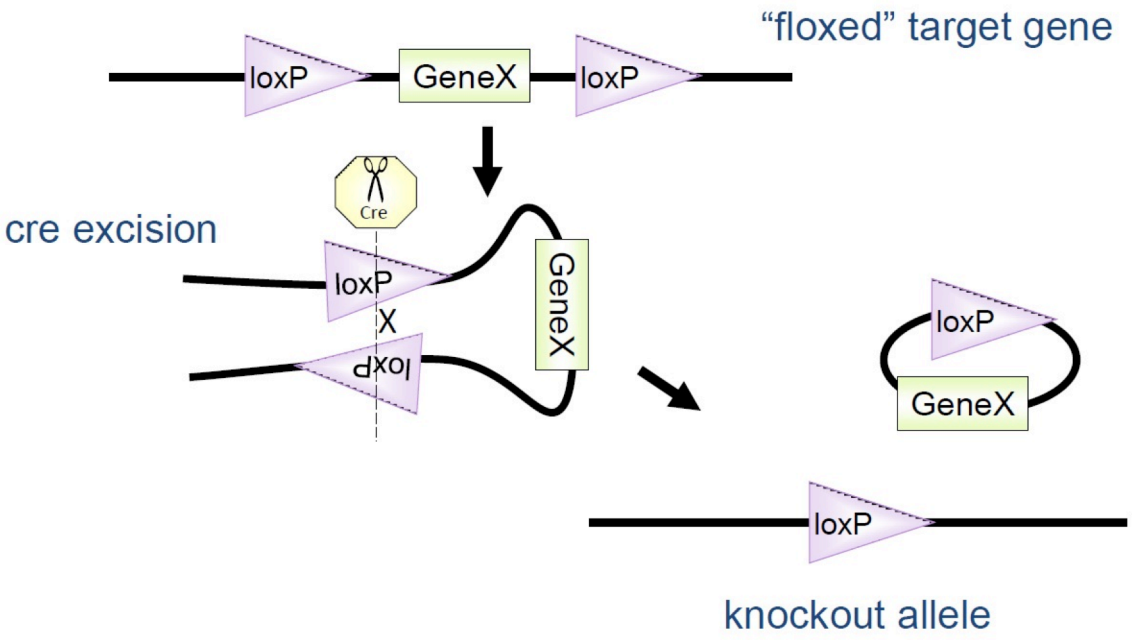

Figure 3. Cre induction results in excision of "floxed" DNA. Cre activity may be induced in mice containing a "floxed" region of DNA as well as a gene for the Cre protein. Once Cre activity is achieved, a single *loxP* site remains in the host genome, while the excised DNA is later targeted for degradation by typical means. Image from Jackson Laboratories.

in safflower oil (0.25 mg tamoxifen/kg body weight). Injections were performed four times for each mouse, once every two days, and mice were allowed at least 14 days for recovery, following tamoxifen administration, prior to gene analysis. As mice exhibited extensive wasting by Cre-induction, individuals with substantial weight loss threatening sustained vigor were analyzed prior to the full recovery time.

Tissue PCR analysis of *Ano1* following Cre induction

After recovery from injection, each mouse was sacrificed, and DNA was extracted from separate tissues of the gastrointestinal tract using the HotSHOT technique, as mentioned above (BioTechniques 2000). Different isolated tissues included the colon and small intestine (SI) – whole intestine, mucosa, or vascular smooth muscle (VSM). In order to determine excision had occurred, the $Ano1^{lox/lox}$ locus was then analyzed as mentioned above.

Real-time quantitative reverse transcription PCR (qRT-PCR) analysis

To quantify the extent of *Ano1* knockdown in Cre-induced mice, RNA was isolated from the hearts and gastrointestinal tissues of an injected $Ano1^{lox/lox}$cre-ERT2^{+} mouse and an injected $Ano1^{+/+}$cre-ERT2^{+} mouse as a negative control. cDNA was synthesized via reverse transcription with qScript (Quanta Biosciences, Inc.), according to manufacturer's protocol. qRT-PCR was then performed in duplicate and analyzed for *Ano1* (NM_001242349) expression using the standard curve method (Bookout, et al., 2006), with normalization to *Gapdh* (NM_008084). Fold change in *Ano1* expression was calculated by comparison to the control mouse.

Ano1$^{lox/\Delta}$cre-ERT2^{+} mice

As Cre-mediated excision of two alleles proved to be inconsistent in gastrointestinal tissues, *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice were bred to better facilitate *Ano1* knockdown (where Δ refers to a null allele) (Figure 1). Cre-induction in these mice would entail excision of only a single allele, perhaps resulting in increased *Ano1* knockdown. To achieve this genotype, *Ano1*$^{lox/lox}$cre-ERT2^{+} mice were bred with *Ano1* heterozygotes (*Ano1*$^{+/\Delta}$), so that a fraction of the offspring contained the *Ano1*$^{lox/\Delta}$cre-ERT2^{+} genotype. These mice contained a single "floxed" *Ano1* allele, as well as a conventional null allele from the heterozygous parent. These mice were then analyzed with PCR, as mentioned above.

Results

Ano1 excision in *Ano1*$^{lox/lox}$cre-ERT2^{+} mice

Following Cre-induction in *Ano1*$^{lox/lox}$cre-ERT2^{+} mice, the *Ano1* locus was analyzed as described above. According to the *Ano1*$^{lox/lox}$ construct (Figure 2), a single 150-bp PCR fragment is indicative of alleles with successful recombination. However, while the desired PCR fragments were observed, detection of additional fragments indicated that complete knockout had not occurred (Figure 4a). Additionally, incomplete *Ano1* excision was corroborated by analysis of specific tissues of an injected *Ano1*$^{lox/lox}$cre-ERT2^{+} mouse, including the heart, and the mucosa and VSM of the SI (Figure 4b). Subsequent tissue-specific qRT-PCR analysis revealed significant *Ano1* knockdown in the heart, whole SI, and SI mucosa, while insignificant knockdown was observed in the SI vasculature (Figure 4c). Immunofluorescence imaging confirmed knockdown of ANO1 on the protein level (Figure 5).

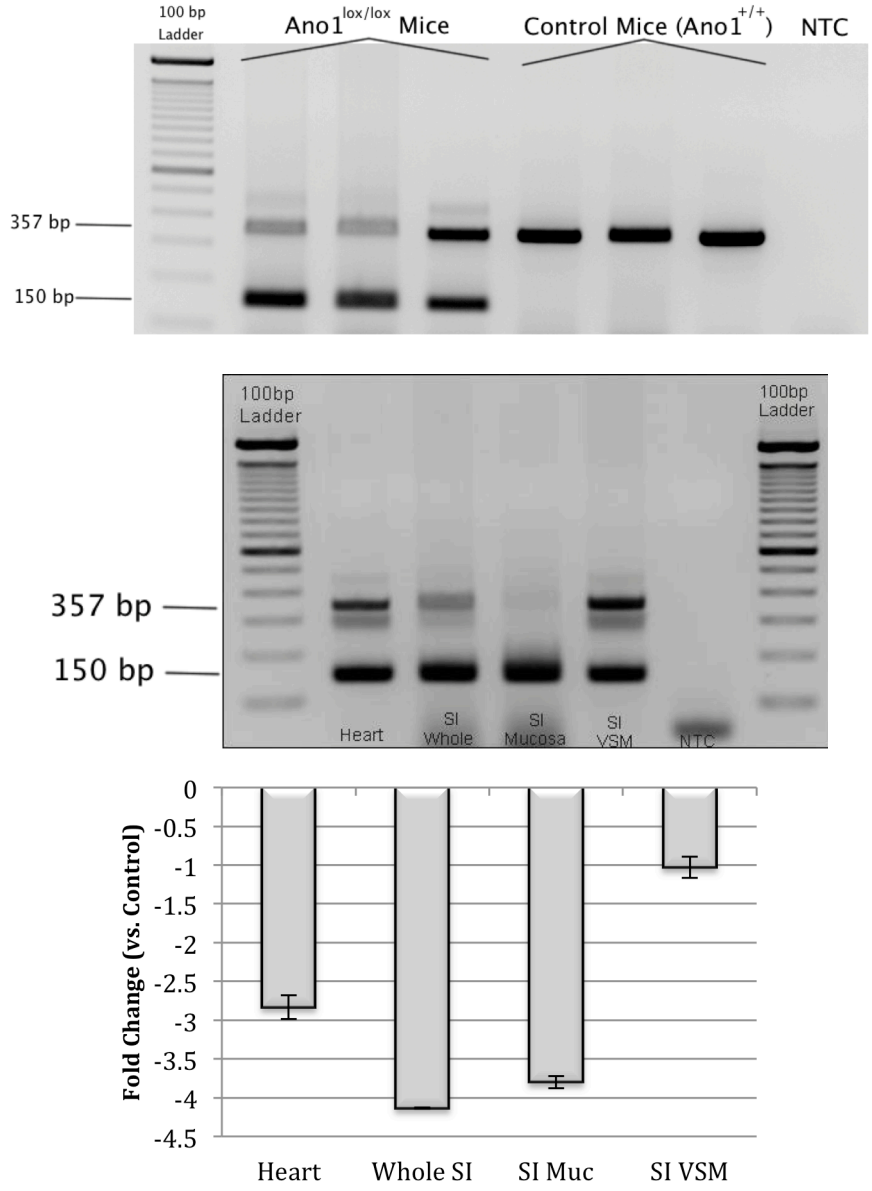

Figure 4. Cre induction in *Ano1*$^{lox/lox}$cre-ERT2^{+} mice yields incomplete *Ano1* excision. **(a)** DNA was isolated from whole SIs of three *Ano1*$^{lox/lox}$cre-ERT2^{+} mice following Cre induction. Following PCR and gel electrophoresis, the observed bands at 150 bp in all three mice indicated that Cre recombination did indeed occur. However, a band at 357 bp, indicating unsuccessful excision, was also observed in the SIs of these mice as well as in the SIs of injected wildtype control mice. This fragment was not observed in the non-template control (NTC). **(b)** Various tissues from a Cre-induced mouse were analyzed as in Figure 3a, showing incomplete excision in various cell types. **(c)** qRT-PCR was performed with cDNA from various tissues of a Cre-induced *Ano1*$^{lox/lox}$cre-ERT2^{+} mouse, and the fold change of *Ano1* was compared to a Cre-induced control mouse (*Ano1*$^{+/+}$cre-ERT2^{+}), following normalization to *Gapdh*. Significant knockdown (< -2.0 fold change) was observed in the heart, whole SI, and SI mucosa, with an insignificant knockdown in the vasculature.

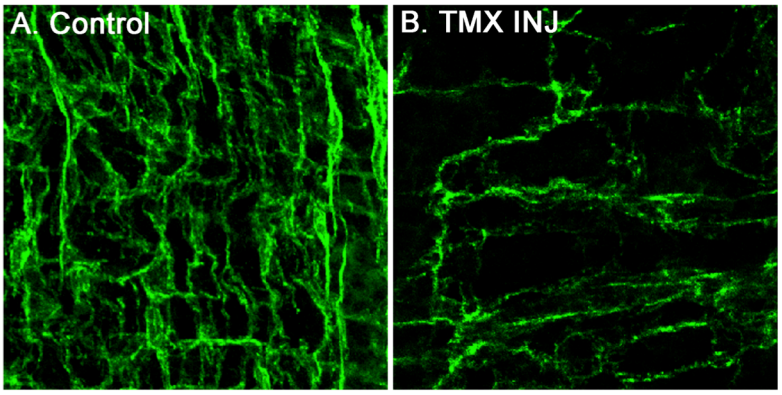

Figure 5. Immunofluorescence examination of ANO1 protein distribution reveals extent of knockdown in induced *Ano1*$^{lox/lox}$Cre-ERT2^{+} mice. Immunofluorescence imaging was used to visualize ANO1 in the small intestine of injected *Ano1*$^{+/+}$Cre-ERT2^{+} control (A) and *Ano1*$^{lox/lox}$Cre-ERT2^{+} (B) mice. In both images, ANO1 has been conjugated to enhanced green fluorescent protein (eGFP). While substantial knockdown was observed, substantial ANO1 expression remains for induced mice.

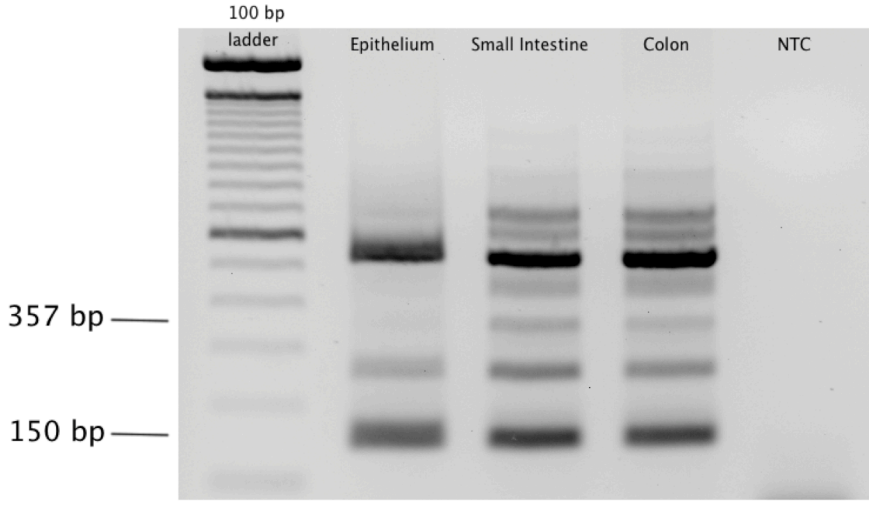

Figure 6. Cre induction in *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice yields incomplete *Ano1* excision. DNA was analyzed from *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice as in Figures 3 and 4. The band at 150 bp, corresponding to the null allele, was observed in the epithelium, SI and colon, but the fragment at 357 bp, corresponding to incomplete excision, was observed in the SI and colon. This indicates that some GI cells still exhibited no recombination of "floxed" *Ano1* alleles. The extraneous bands observed correspond to recombination of portions of the knockout allele already present, and are unrelated to Cre activity.

Ano1 excision in *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice

Cre activity was induced in *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice as previously discussed, and various tissues were analyzed for *Ano1* knockdown (Figure 6). Using this breeding scheme, knockdown was observed in the epithelium, SI, and colon. The presence of 357bp-sized fragments in the SI and colon, however, indicates that *Ano1* excision is still incomplete in the gastrointestinal tract.

Discussion and Conclusions

Incomplete Knockout of *Ano1*

Cre induction in both *Ano1*$^{lox/lox}$cre-ERT2^{+} and *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice resulted in an incomplete *Ano1* knockout in gastrointestinal tissues, with lack of recombination observed especially in the VSM of the SI (Figures 4-6). This incomplete Cre activity is potentially due to mosaic Cre expression, particularly within the gastrointestinal tract. Mosaic expression is further evidenced by the different extents of knockdown observed in separate tissues; for instance, Cre excision seems to be completely successful in the epithelium (Figure 6). Mosaic Cre expression has been identified in the past as a limiting factor for gene deletions, as Cre protein activity is not observed consistently in all tissues and cell types (Ruzankina, et al. 2007, Bao, et al. 2013). In particular, although it is ubiquitin-controlled, the specific Cre strain in this study does appear to be characterized by mosaic expression in the gastrointestinal tract, possibly explaining the incomplete recombination observed (Figure 7). Furthermore, the observed expression pattern provides evidence for incomplete knockdown in the SI vasculature (Figure 4c). Engineering a novel Cre strain that is tissue-specific to the gastrointestinal tract could possibly overcome this issue.

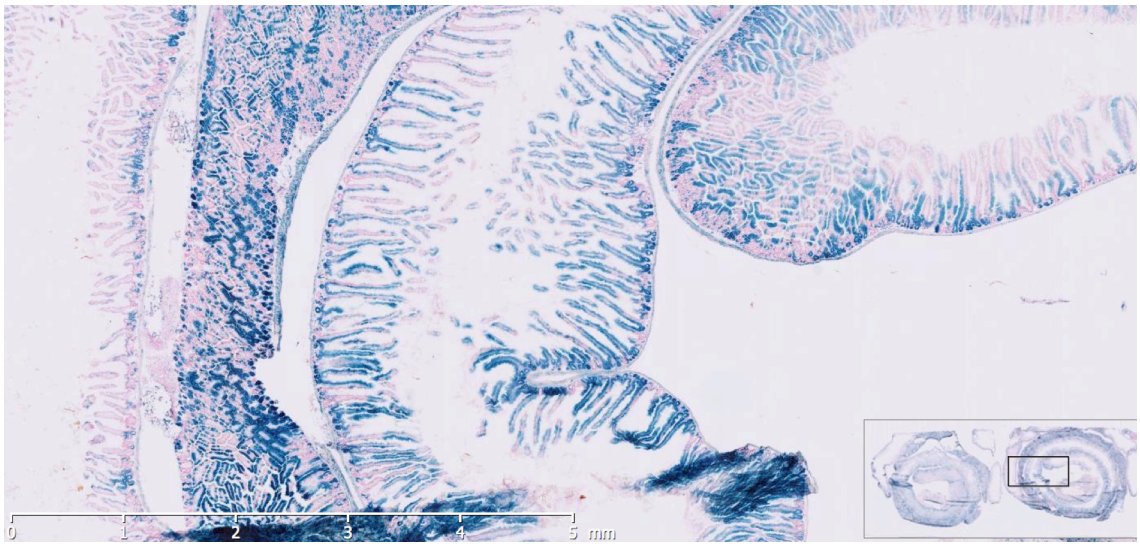

Figure 7. Mosaic Cre expression in the SI for B6.Cg-Tg(UBC-cre/ESR1)1Ejb/J strain (Jackson Laboratories). The B6.Cg-Tg(UBC-cre/ESR1)1Ejb/J strain exhibits mosaic Cre expression in the murine SI. The figure depicts the use of this specific Cre strain to cause recombination of a beta-galactosidase reporter. With this system, Cre recombination results in a blue coloration, while the pink cells correspond to cells in which Cre activity was not observed. This image was obtained from Jackson Laboratories' strain-specific information sheet (http://cre.jax.org/UBC-creERT/UBC-creERT.html).

Future Directions

Germline *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice. An additional strategy was to induce Cre activity in *Ano1*$^{lox/lox}$cre-ERT2^{+} mice as above. Rather than being subjected to the above DNA analysis methods, these induced mice were immediately bred with *Ano1*$^{lox/lox}$cre-ERT2^{+} mice. Assuming successful Cre-mediated excision of the germ cells of the induced parent, then a fraction of the offspring would contain a null allele from the induced parent, and a "floxed" allele from the other parent. Mice with this genotype are comparable to the *Ano1*$^{lox/\Delta}$cre-ERT2^{+} mice described above, except that the null allele

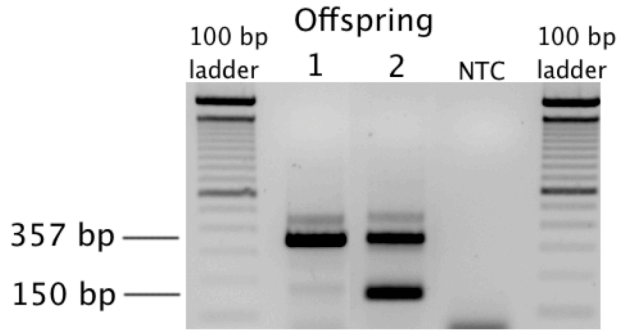

Figure 8. Germline *Ano1*$^{lox/\Delta}$cre-ERT2^{+} offspring may be easily distinguished by genotyping methods. Epithelial tissue was obtained from germline *Ano1*$^{lox/\Delta}$cre-ERT2^{+} offspring, bred from a Cre-induced *Ano1*$^{lox/lox}$cre-ERT2^{+} mouse and a non-induced *Ano1*$^{lox/lox}$cre-ERT2 mouse. These tissues were genotyped as described. Both offspring contain fragments at 357 bp, corresponding to an unaffected "floxed" allele. Offspring 2, however contains a fragment at 150 bp, corresponding to an excised *Ano1* allele. This offspring therefore descended from an affected germ cell in the Cre-induced parent.

originates from Cre-mediated excision in a prior generation, rather than from a conventional knockout allele. These offspring could then be induced and analyzed as before. Unfortunately, this technique is more difficult to accomplish, since germline manipulation is often unpredictable, and only one germ cell is carried on to further generations. Thus far, offspring of the desired genotype have been obtained, and they may be easily distinguished (Figure 8). Cre activity will be induced for these offspring to determine if excision is more successful. In theory, Cre induction in these mice would be substantially more effective than for the *Ano1*$^{lox/lox}$ genotype, since the Cre recombinase must only affect one allele. Expected recombination efficiency would likely compare with that of the *Ano1*$^{lox/\Delta}$ genotype. A germline-focused approach, however, would ensure that both alleles are being excised using the same technology (Cre/loxP recombination), even if one allele is knocked out in a prior generation. Furthermore,

conventional *Ano1* Δ alleles – those not excised by Cre/loxP recombination – contain a number of DNA sites closely resembling the *loxP* sequence. These sites may be subject to Cre/loxP recombination, possibly affecting physiology in a tangible way. The germline-focused approach would correct for this issue. With either approach, mosaic Cre expression could still preclude the possibility of a full knockout, as the specific Cre strain used in this study is not active in all cells in the gastrointestinal tract (Figure 7).

Further quantification of *Ano1* expression. Real-time qRT-PCR and immunofluorescence imaging have yet to be performed on the gastrointestinal tissues of injected $Ano1^{lox/\Delta}$ mice. Doing so would quantify the advantage to affecting only a single allele, and would possibly dictate the necessary steps to further optimize recombination efficiency.

Conclusions

A conventional *Ano1*-knockout mouse model has thus far not been of particular use in the study of the function of ANO1, particularly because conventional *Ano1* knockout is particularly deleterious for developing mice. Therefore, it remains difficult to experimentally determine the role of this protein in gastrointestinal ICC of fully developed individuals. Although Cre recombination is an alluring prospect for the induced knockout of *Ano1,* existing Cre strains that are active in the gastrointestinal tract exhibit mosaic gene expression particularly in the SI. Cre-mediated *Ano1* excision with these strains therefore results in incomplete gene deletion, as seen in this study.

Nonetheless, the specific Cre/loxP recombination system implemented was able to result in significant *Ano1* deletion in a number of tissues. A number of different breeding schemes were then implemented that appeared to improve the extent of Cre

activity in the gastrointestinal tract. Further studies are needed to quantify the extent of *Ano1* gene deletion and ANO1 knockdown across these distinct breeding schemes and genotypes. In theory, the extent of ANO1 knockdown will likely correlate with change in physiological function. Studying the role of ANO1 through one of the systems proposed would then allow for a thorough investigation into its function in ICC and the gastrointestinal muscle layer. In particular, such a study would possibly further elucidate the mechanisms by which ICC are able to regulate gastrointestinal smooth muscle contraction and pacemaking, providing a more thorough understanding of gastrointestinal motility and diseases.

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